G-motif Oligonucleotides and Uses thereof

The present invention relates to a composition comprising an oligonucleotide comprising (a) the sequence N_1 - N_2 - G - N_3 - G, wherein N_1 represents any nucleotide if N_2 and N_3 are G; N_2 represents any nucleotide if N_1 and N_3 are G; and N_3 represents any nucleotide if N_1 and N_2 are G, or (b) the sequence of (a), wherein at least one nucleotide is replaced by a corresponding nucleotide analog or derivative. The present invention also relates to an oligonucleotide having (a) a sequence selected from the group consisting of the sequences of SEQ ID NOs: 1 to 19, or (b) a sequence of (a), wherein at least one nucleotide is replaced by a corresponding nucleotide analog or derivative. Furthermore, a vector comprising an oligonucleotide of the present invention, a host cell comprising the vector of the present invention, a method for the production of the oligonucleotide of the present invention as well as a kit comprising the composition, the oligonucleotide, the vector, and/or the host cell of the present invention are described. The present invention further relates to the use of the composition, and/or the oligonucleotide of the present invention for the production of a pharmaceutical composition for preventing or treating septic shock, inflammation, autoimmune diseases, T_H1-mediated diseases, bacterial infections, parasitic infections, viral infections, spontaneous abortions, and/or tumors. Also described is the use of the composition, and/or the oligonucleotide of the present invention to inhibit activation of antigen-presenting cells, to inhibit the uptake of DNA by a cell, to stimulate natural killer cells, to co-stimulate cytotoxic T-lymphocytes or natural killer cells, to enhance the production of antibodies directed against an antigen, to enhance the uptake of an agent by a cell, and/or to induce proliferation of bone marrow cells in vitro or in vivo.

It is known that cells of the immune system are exported from the bone marrow and undergo a series of differentiation events which confer upon them the capacity to recognize and control foreign pathogens and cancer cells by discriminating between self versus non-self. These differentiation and education events are tightly controlled by cells surface receptor engagement via intracellular signal transduction and the

milieu of autocrine, paracrine and endocrine soluble ligands, typically referred to as cytokines. Cell to cell interaction occurs in discreet locals such as the thymus, spleen or lymph nodes but also in the periphery. The system thus balances receptor and cytokine input signals to regulate cellular proliferation, differentiation and maturation of immune effector cells (Paul WE, Cell 1989; 57: 521). Pathogen challenge, by supplying danger stimuli, mobilizes the immune system to respond. Most typically these responses result in the clearance of the challenging pathogen but can also result in overshoot or misdirected responses leading to conditions such as allergy or autoimmune diseases. Through outside intervention the immune system can be enhanced, e.g., by vaccination or cytokine therapies, to bolster favorable host responses, or can be suppressed, e.g., by drug intervention or cytokine therapies, to limit unfavorable responses.

The functioning of the immune system is based on two distinct recognition systems: innate (myeloid cells, NK cells etc.) or nonclonal host defense and adaptive (T and B cells) or clonal host defense. The innate immune systems receptors recognize conserved molecular structures shared by a large group of pathogens, termed pattern recognition receptors (PRRs) (reviewed in Fearon DT, Locksley RM, Science 1996; 272: 50; Medzhitov R, Janeway CA, Jr., Curr Opin Immunol 1997; 9: 4). The main difference between PRRs and clonally-distributed antigen receptors of the adaptive system is that their specificities are germline encoded. Thus a parameter imposed on PRRs is recognition of non-self structural patterns. While T and B cell receptors, through their specificity and diversity, represent the crowning achievement of the vertebrate adaptive immune system they do not distinguish self from non-self. The innate system controls the initiation of the adaptive immune response by regulating the expression of costimulatory activity on antigen presenting cells (APCs), and instructs the adaptive immune system to develop a particular effector response (humoral, cytotoxic, Th1 versus Th2) by releasing effector cytokines.

The adaptive immune system of vertebrates (T and B cells) consists of several interacting components. They can be divided into humoral and cellular branches. Humoral immunity involves antibodies, proteins which are produced and secreted by

B cells into the body fluids and which directly recognize an antigen. The cellular system, in contrast, relies on T cells which recognize cells presenting foreign antigens and then responding by proliferation, cytotoxicity or secretion of cytokines. This basic functional division reflects two different strategies of immune defense. Humoral immunity is mainly directed at antigens which are whole proteins, the cellular system responds to antigens which are actively processed and presented by either APC or pathogen infected cells (see, e.g., Paul WE, Cell 1989; 57: 521).

Antibody molecules, the effectors of humoral immunity, are secreted by B cells in response to antigen receptor stimulation, co-receptor stimulation and cytokines. Antibodies can bind to and inactivate antigen directly (neutralizing antibodies) or activate other cells of the immune system to destroy the antigen depending on isotype; IgM, IgG1, etc. Isotype class switching in B cells is controlled by the cytokine milieu. Abnormalities in antigen response, co-receptor engagement or cytokine milieu can lead to suboptimal immune responses, tolerance or autoimmunity. In addition the cytokine milieu can push the isotype repertoire to reflect either Th1 (IgG2) or Th2 (IgG1 and IgE) responses which can have beneficial or detrimental effects dependent on the target antigen and challenge source.

Cellular immune recognition is mediated by a special class of lymphoid cells, T cells. These cells do not recognize whole antigens but instead they respond to degraded peptide fragments thereof which appear on the surface of the target cell bound to proteins called major histocompatibility complex (MHC) molecules. Essentially all nucleated cells have MHC class I molecules whereas MHC II are restricted to immune cells with special presenting qualities.

Proteins produced within the cell are continually degraded to peptides as part of normal cellular metabolism. These fragments are bound to the MHC I molecules and are transported to the cell surface. Thus the cellular immune system is constantly monitoring the spectra of proteins produced by all cells in the body and is poised to eliminate any cells producing foreign antigens or abnormal self-antigens. This is a function of cytolytic T cells. Additionally, APCs take up antigens from the

environment, process them to peptides, and present them in the context of MHC II to helper T cells (Th cells) which respond by producing cytokines. These responses can be divided into Th1 or Th2, as defined by the cytokine pattern. Th1 or Th2 responses are generated dependent on the pathogen challenge and/or the contextual engagement through which the response is generated. Again, Th1 or Th2 biased responses can have either beneficial or detrimental outcomes dependent on the target antigen and the intensity of response.

APCs, such as dendritic cells and macrophages, represent a decisive interface between the innate and adaptive immune system (Banchereau J, Steinman RM, Nature 1998; 392: 245). By virtue of their surveillance and phagocytic/endocytic properties these cells engage through PRRs pathogens at an early stage in infectious challenge. Signals induced by pattern recognition can be grouped into three categories: first, inflammatory responses including IL-1, TNF, IL-6, type I IFN and chemokines; second, costimulators of T cell activation including B7.1 (CD80), B7.2 (CD86) and CD40; and third, effector cytokines including IL-10, IL-12, TNF-α and IFN-γ. Before a productive response can ensue, APCs need first to be activated thus acquiring the ability to instigate productive T and B cell activation through the above mentioned mediators.

Tumor necrosis factor (TNF) produced by activated macrophages exerts a key role in the cytokine network with regard to the pathogenesis of many infectious and inflammatory diseases (reviewed in Eigler A, Sinha B, Hartmann G, Endres S, Immunol Today 1997; 18: 487). Synthesis of TNF is stimulated in myeloid cells by many different exogenous substances such as LPS, β-glucanes and foreign DNA, or by endogenous mediators such as IL-1 which is also induced by foreign DNA. High concentrations of TNF have been demonstrated in a variety of diseases: infectious (e.g., sepsis syndrome, bacterial meningitis, cerebral malaria, and AIDS), autoimmune (e.g., rheumatoid arthritis, Crohn's disease, sarcoidosis, multiple sclerosis, Kawasaki syndrome, graft-versus host disease and transplant rejection), and organ failure (e.g., adult respiratory distress syndrome), but also in therapies: yellow fever vaccination.

Acute release of toxic amounts of TNF-α can also follow bacterial DNA challenge in vivo leading to lethal toxic shock in mouse models (Sparwasser T, Miethke T, Lipford G, et al., Nature 1997; 386: 336; Sparwasser T, Miethke T, Lipford G, et al., Eur J Immunol 1997; 27: 1671). These attributes are similar to previously described PRR signaling induced by the bacterial product lipopolysaccaride (LPS). Bacterial DNA can also sensitize mice for the action of LPS and more importantly, bacterial DNA or oligonucleotides (in the following abbreviated by the letters "ODN") comprising (a) CpG-dinucleotide(s) synergise with LPS in inducing macrophage cytokines in vitro and toxic amounts of TNF-α in vivo (Sparwasser T, Miethke T, Lipford G, et al., Eur J Immunol 1997; 27: 1671; Cowdery JS, Chace JH, Yi AK, Krieg AM, J Immunol 1996; 156: 4570). Acute systemic stimulation resulting in overproduction of proinflammatory cytokines, that cause toxic shock and other infectious and autoimmune syndromes, unquestionably is the worst scenario for bacterial infections. Thus, there exists a need in the art for a specific, cheap, and easily producible means for inhibiting the release of detrimental amounts of TNF-α.

In addition, chronic or redirected Th1 response patterns (reviewed in Mosmann TR, Sad S, Immunol Today 1996; 17: 138; Trembleau S, Germann T, Gately MK, Adorini L, Immunol Today 1995; 16: 383) have been demonstrated in a variety of diseases: infectious (e.g., streptococcal induced arthritis, and Lyme arthritis), and autoimmune (e.g., rheumatoid arthritis, chronic inflammatory bowel disease, psoriasis vulgaris, experimental allergic encephalomyelitis (EAE), insulin-dependent diabetes mellitus (IDDM), spontaneous abortion, graft-versus host disease and transplant rejection). Th2 responses are beneficial in various helminth infection and bacterial responses. Thus, in the context of vaccination the ability to direct responses toward Th2 for certain diseases would be beneficial.

Vaccination is the process of preparing an animal to respond to an antigen. Vaccination is more complex than immune recognition and involves not only B cells and cytotoxic T cells but other types of lymphoid cells as well. During vaccination, cells, which recognize the antigen (B cells or cytotoxic T cells), are clonally

expanded. In addition, the population of ancillary cells (helper T cells which provide co-receptor and cytokine stimulation) specific for the antigen also increase. Vaccination also involves specialized antigen presenting cells, which can process the antigen and display it in a form, which can stimulate one of the two pathways (macrophages and dendritic cells).

A foreign antigen is introduced into an animal where it activates specific B cells by binding to surface immunoglobulins. It is also taken up by antigen processing cells, wherein it is degraded, and appears in fragments on the surface of these cells bound to Class II MHC molecules. Peptides bound to class II molecules are capable of stimulating the helper class of T cells. Both helper T cells and activated B cells are required to produce active humoral immunization. Cellular immunity is stimulated by a similar mechanism but entry into the MHC I presentation pathway of antigen presenting cells is typically by intracellular pathogen replication and not achieved by injection of protein antigen only.

Standard vaccination schemes nearly always produce a humoral immune response. The humoral system protects a vaccinated individual from subsequent challenge from a pathogen and can prevent the spread of an intracellular infection if the pathogen goes through an extracellular phase during its life cycle; however, it can do relatively little to eliminate intracellular pathogens. Cytotoxic immunity complements the humoral system by eliminating the infected cells and cancer cells. Thus effective vaccination should activate both types of immunity.

As mentioned above, a cytotoxic T cell response is necessary to remove intracellular pathogens such as viruses as well as malignant cells. However, a major problem exists in the prior art with respect to the presention of an exogenously administered antigen in adequate concentrations in conjunction with Class I molecules to assure an adequate response.

Furthermore, directed and elevated immune responses to antigens can be achieved by the use of adjuvants and/or delivery vehicles. The term „immune adjuvant" refers

to compounds which when administered to an individual or tested in vitro, increase the immune response to an antigen.

A variety of immune adjuvants are known in the art. These include classical adjuvants such as complete Freund's adjuvant or more recently developed compounds like liposomes. Many of these adjuvants, however, have adverse side effects such as local inflammation after administration, or their production is time consuming and/or requires complicated standardized production protocols, in particular when vaccination of humans is envisaged.

As with the above discussed immune modulators, it would be desirable to have an adjuvant available that is, when properly administered, devoid of harmful side effects. Such a compound should, moreover, require only low costs of production for being universally applicable. Optimally, such a compound should allow the modulation of the immune system or the above recited branches of the immune system in various beneficial ways.

The solution of the above outlined technical problem underlying the present invention, namely the provision of such a compound, is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a composition comprising an oligonucleotide comprising

(a) the sequence: $N_1 - N_2 - G - N_3 - G$,
wherein N_1 represents any nucleotide if N_2 and N_3 are G; N_2 represents any nucleotide if N_1 and N_3 are G; and N_3 represents any nucleotide if N_1 and N_2 are G; or

(b) the sequence of (a), wherein at least one nucleotide is replaced by a corresponding nucleotide analog or derivative.

The term "composition", as used in accordance with the present invention, denotes a solution, e.g., a buffer, comprising at least one of the oligonucleotides of the present invention. Alternatively, the term "composition" denotes a formulation comprising at

least one of the oligonucleotides of the present invention and at least one further compound. Said formulation may be of liquid, solid or vaporous nature.

As used in accordance with the present invention, the term "nucleotide analog or derivative" denotes any nucleoside phosphate the nucleoside of which deviates in its chemical structure from the nucleosides guanosine, adenosine, thymidine, uridine or cytidine. Such modified nucleosides are well known to the person skilled in the art and comprise, e.g., 5,6-dihydrouridine, ribothymidine, inosine or 1-methylguanosine (see, e.g. Lewin B, Genes, 1983, John Wiley & Sons, Inc., NY). In accordance with the present invention, one or more nucleotides of the oligonucleotides of the present invention or the oligonucleotides to be employed in accordance with the composition of the present invention may be replaced by said nucleotide analogs and/or derivatives as long as the modified oligonucleotides remain functionally equivalent to their unmodified counterparts, i.e. as long as they maintain essentially the same portfolio of biological activities that are described in accordance with this invention. "Essentially the same portfolio of biological activities" means that the modified oligonucleotides described above display at least one of the biological activities described in accordance with this invention. Alternatively or additionally, the different biological activities of the modified oligonucleotides may be more or less pronounced compared to the corresponding activities of their unmodified counterparts.

As mentioned above, a potential deleterious effect of foreign DNA and CpG motif(s) containing oligonucleotides derived thereof is the activation of myeloid lineage immune cells and/or lymphoid lineage cells to produce toxic cytokines. In addition to toxic cytokines, the strong Th1 inducing potential of foreign DNA can hyperactivate the immune system leading to potential autoreactivity and tissue damage.

In accordance with the present invention, it was surprisingly found that the oligonucleotides of the present invention and/or the oligonucleotides to be employed in accordance with the composition of the present invention have the potential to efficiently block the activating effects on antigen-presenting cells of foreign DNA or CpG motif(s) containing oligonucleotides derived therefrom. Without wanting to be bound to a specific scientific theory, the experiments performed in accordance with the present invention suggest that foreign DNA uptake is receptor mediated and that

blockade of this cell surface DNA receptor with the oligonucleotides of the present invention or the oligonucleotides to be employed in accordance with the composition of the present invention blocks the CpG driven cell activation.

Furthermore, it was unexpectedly found in accordance with the present invention that these oligonucleotides also elicit costimulatory signals leading to lymphocyte activation in the absence of myeloid cell activation. Thus, T cell costimulation by the oligonucleotides of the present invention and/or the oligonucleotides to be employed in accordance with the composition of the present invention may be advantageously used to promote T cell activation in the apparent absence of APCs, thus circumventing the need of APC mediated cross priming. T cell costimulation mediated by the oligonucleotides of the present invention or the oligonucleotides to be employed in accordance with the composition of the present invention also allows induction of CTLs in vivo. This may allow for the induction of immune responses against tumor cells expressing tumor antigen but lacking co-stimulatory molecules.

Thus, it is envisaged in accordance with the present invention that the oligonucleotides of the present invention and/or the oligonucleotides to be employed in accordance with the composition of the present invention may be used to beneficially influence the overall cytokine production of the cells of the immune system in that the production of (co-)stimulatory cytokines is enhanced whereas the production of inflammatory cytokines like, e.g., TNF-α is suppressed.

As will be readily appreciated by the person skilled in the art, a further advantage of the oligonucleotides of the present invention and/or the oligonucleotides to be employed in accordance with the composition of the present invention is that, especially if chemically synthesized, they represent compounds which can be easily produced at low costs. Moreover, due to the chemical production, the apparent absence of, e.g., contaminating pathogens, allergens or otherwise harmful agents makes the oligonucleotides of the present invention and/or the oligonucleotides to be employed in accordance with the composition of the present invention superior compounds when used as pharmaceutical compositions, for the preparation of a pharmaceutical composition or in methods for the treatment of diseases.

In a preferred embodiment of the composition of the present invention

(a) N_1 represents G > A > T/U > C if N_2 and N_3 are G; N_2 represents G > A > T/U > C if N_1 and N_3 are G; and N_3 represents G > A > T/U > C if N_1 and N_2 are G; or

(b) N_1, N_2, and N_3 represent a nucleotide analog or derivative of the nucleotides of (a).

The term "G > A" cited above refers to the fact that Gs in the mentioned position usually show an improved biological effect over As. Accordingly, Gs are more preferred in said position than As. This rule mutatis mutandis applies to the above-referenced further relationships between nucleotides.

As could be shown in accordance with the present invention, oligonucleotides are most effective if the positions N_1, N_2, and/or N_3 are occupied by Gs. Adenosine phosphates are almost equally preferred. Less preferred are T/Us, and least preferred are Cs.

In another preferred embodiment of the composition of the present invention said oligonucleotide comprises

(a) the sequence GGGGG, GAGGG, GGGAG, GTGGG or GGGTG; or

(b) a sequence of (a), wherein at least one nucleotide is replaced by a corresponding nucleotide analog or derivative.

In a further preferred embodiment of the composition of the present invention said oligonucleotide has

(a) a sequence selected from the group consisting of the sequences of SEQ ID NOs: 1 to 19; or

(b) a sequence of (a), wherein at least one nucleotide is replaced by a corresponding nucleotide analog or derivative.

In a still further preferred embodiment of the composition of the present invention said oligonucleotide consists of between 10 and 50 nucleotides.

In a more preferred embodiment of the composition of the present invention said oligonucleotide consists of between 13 and 30 nucleotides.

In a most preferred embodiment of the composition of the present invention said oligonucleotide consists of between 17 and 21 nucleotides.

In another preferred embodiment of the composition of the present invention the G-rich pentamer motif as defined above represents the 3'-terminus of said oligonucleotide.

The present invention also relates to an oligonucleotide consisting of

(a) a sequence selected from the group consisting of the sequences of SEQ ID NOs: 1 to 19; or

(b) a sequence of (a), wherein at least one nucleotide is replaced by a corresponding nucleotide analog or derivative.

In a preferred embodiment of the composition or the oligonucleotide of the present invention, the nucleotides of said oligonucleotide are linked via phosphodiester-, phosphorothioate-, methylphosphonate- or peptide bonds.

Thus, the oligonucleotides of the present invention and/or the oligonucleotides to be employed in accordance with the composition of the present invention may be peptide nucleic acids (PNAs).

The linkage of the nucleotides of the oligonucleotides of the present invention and/or the oligonucleotides to be employed in accordance with the composition of the present invention via bonds other than phosphodiester bonds may increase their resistance against nucleases. Especially if used as pharmaceutical composition, in the preparation of a pharmaceutical composition or in a method for the treatment of a disease, the nucleotides may be advantageously linked via nuclease-resistant bonds which increase the half-life of the corresponding oligonucleotide in, e.g., the organism. This may, in turn, decrease the frequency of administration of the pharmaceutical composition and, thus, may, e.g., contribute to a patient's comfort.

In a further preferred embodiment of the composition or the oligonucleotide of the present invention said oligonucleotide is DNA or RNA.

The oligonucleotides of the present invention and/or the oligonucleotides to be employed in accordance with the composition of the present invention may be DNA, cDNA, RNA or (semi)synthetically produced DNA or RNA or a recombinantly produced chimeric nucleic acid molecule.

In a still further preferred embodiment of the composition or the oligonucleotide of the present invention said oligonucleotide is single-stranded.

In another preferred embodiment the composition of the present invention is a pharmaceutical composition optionally comprising a pharmaceutically acceptable carrier and/or diluent.

Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical, intradermal, intranasal or intrabronchial administration. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. A typical dose can be, for example, in the range of 0.001 to 1000 µg (or of nucleic acid for expression or for inhibition of expression in this range); however, doses below or above this exemplary range are envisioned, especially considering the aforementioned factors. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 µg to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1 µg to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately 10^6 to 10^{12} copies of the

DNA molecule. The compositions of the invention may be administered locally or systemically. DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Furthermore, the pharmaceutical composition of the invention may comprise further agents depending on the intended use of the pharmaceutical composition.

In a particularly preferred embodiment the composition of the present invention is a vaccine.

As mentioned above, the adequate presentation of exogenously administered antigen in the context of MHC class I molecules has imposed on the person skilled in the art severe problems. However, due to their CTL stimulating properties, the oligonucleotides of the present invention and/or the oligonucleotides to be employed in accordance with the composition of the present invention may advantageously be used in the development of vaccines against tumor-specific antigens (e.g., on breast or colon cancer cells), and against weakly immunogenic viral proteins (e.g., HIV, Herpes, non-A, non-B hepatitis, CMV and EBV).

It is also envisaged in accordance with the present invention to use the oligonucleotides to provide a cellular immune response alone in immunizing against agents such as viruses for which antibodies have been shown to enhance infectivity. In addition, the oligonucleotides of the present invention may advantageously be

used to provide such a response against both chronic and latent viral infections and against malignant cells.

Furthermore, the oligonucleotides of the present invention and/or the oligonucleotides to be employed in accordance with the composition of the present invention may be used to efficiently enhance the immune response of an individual against an antigen which is weakly immunogenic when administered alone or which is toxic at the concentration which evokes an immune response.

It is, moreover, envisaged in accordance with the present invention to utilize the adjuvant effect of the oligonucleotides to lower the dose of antigen necessary to achieve an immune response by enhancing presentation, influence the cytokine milieu or alter co-receptor expression on antigen presenting cells.

As regards the application routes, dosages, formulations, etc. of the vaccines of the present invention, essentially the same applies that has been discussed above in connection with the pharmaceutical compositions of the present invention.

In another embodiment the present invention relates to a vector comprising an oligonucleotide of the present invention.

The vector of the present invention may be, e.g., a plasmid, cosmid, virus, bacteriophage or another vector used conventionally in genetic engineering, and may comprise further genes such as marker genes which allow for the selection of said vector in a suitable host cell and under suitable conditions.

In still another embodiment the present invention relates to a host cell comprising the vector of the present invention.

The host cell can be any prokaryotic or eukaryotic cell, such as a bacterial, preferably Escherichia coli, insect, fungal, preferably Saccharomyces cerevisiae, plant, animal or human cell.

The present invention also relates to a method for the production of the oligonucleotide of the present invention, said method comprising the steps of culturing the host cell of the present invention under conditions that cause production of the oligonucleotide, and recovering said oligonucleotide from the culture.

Alternatively and preferably, of course the oligonucleotide of the present invention or the oligonucleotide to be employed in accordance with the composition of the present invention may be chemically synthesized according to methods well known in the art (Finnan et al. Nucleic Acids Symp Ser 7, 1980, 133-145), or may be a (semi)synthetic nucleic acid. As used in accordance with the present invention, the term "(semi)synthetic" denotes oligonucleotides which, after their chemical synthesis, have been, e.g., enzymatically modified. Such modifications are well known in the art and comprise, e.g., the introduction of methyl residues into the oligonucleotide. The term "(semi)synthetic" also denotes oligonucleotides which comprise a chemically and a biologically synthesized part.

The present invention further relates to an oligonucleotide obtainable by the method of the present invention.

In another embodiment the present invention relates to a kit comprising the composition, the oligonucleotide, the vector, and/or the host cell of the present invention.
The components of the kit of the invention may be packaged in containers such as vials, optionally in buffers and/or solutions. If appropriate, one or more of said components may be packaged in one and the same container.

In still another embodiment the present invention relates to the use of the composition, and/or the oligonucleotide of the present invention, and/or the oligonucleotide to be employed in accordance with the composition of the present invention for the production of a pharmaceutical composition for preventing or treating septic shock, inflammation, autoimmune diseases, T_H1-mediated diseases, bacterial infections, parasitic infections, viral infections, spontaneous abortions, and/or tumors.

In a further embodiment the present invention relates to a method for preventing or treating septic shock, inflammation, autoimmune diseases, T_H1-mediated diseases, bacterial infections, parasitic infections, viral infections, spontaneous abortions,

and/or tumors comprising administering to a subject the composition, and/or the oligonucleotide of the present invention, and/or the oligonucleotide to be employed in accordance with the composition of the present invention.

In a preferred embodiment of the use or the method of the present invention said septic shock is induced by DNA, preferably of non-vertebrate origin, said autoimmune diseases are rheumatoid arthritis, Crohn's disease, sarcoidosis, multiple sclerosis, Kawasaki syndrome, graft-versus-host disease, and/or transplant rejection, said T_H1-mediated diseases are streptococcal induced arthritis, Lyme arthritis, chronic inflammatory bowel disease, psoriasis vulgaris, experimental allergic encephalomyelitis (EAE), and/or insulin-dependent diabetes mellitus (IDDM), said parasitic infections are Leishmaniasis or Toxoplasmosis, and/or said viral infections are Cytomegalovirus- and/or HIV-infection.

Septic shock is inducible not only by DNA but also by, e.g., LPS and superantigens. Interestingly, it was found in accordance with the present invention that septic shock induced by LPS or superantigens can not be blocked by the oligonucleotides of the present invention. However, it is well known in the art that different inducers of septic shock act in a synergistic fashion. Therefore, it is also envisaged in accordance with the present invention that the oligonucleotides of the present invention may be used to suppress the synergistic action of DNA and a second inducer of septic shock.

It was further found in accordance with the present invention that the time point of administration is not crucial for the oligonucleotides of the present invention to effectively block septic shock. Thus, the oligonucleotides of the present invention are superior to known blocker of septic shock like, e.g., anti-TNF antibodies where the time point of administration is not only of extraordinary importance but may decide whether the treatment results in a positive or detrimental outcome.

In another embodiment the present invention relates to the use of the composition, and/or the oligonucleotide of the present invention, and/or the oligonucleotide to be employed in accordance with the composition of the present invention to inhibit activation of antigen-presenting cells.

In a preferred embodiment of the use of the present invention said antigen-presenting cells are macrophages, dentritic cells and/or B-lymphocytes. Macrophages. dendritic cells and B cells (antigen presenting cells) utilizing PRRs recognize non-self DNA through CpG motifs and initiate inflammatory responses. In early work by Yamamoto et al. it was concluded that IFN-α/β produced by immunostimulatory ODN stimulated spleen cells possibly came from an adherent cell population (Yamamoto S, Kuramoto E, Shimada S, Tokunaga T., Jpn J Cancer Res 1988; 79: 866). We and others discovered that DNA from gram negative and gram positive bacteria, plasmid DNA or synthetic CpG-ODN sequence dependently triggered macrophages to activate the transcription factor NFkB, to transcribe cytokine mRNAs and secrete the proinflammatory cytokines, TNF-α, IL-1. IL-6 and IL-12 (Stacey KJ, Sweet MJ, Hume DA, J Immunol 1996; 157: 2116, Sparwasser T, Miethke T, Lipford G, et al., Nature 1997; 386: 336, Sparwasser T, Miethke T, Lipford G, et al., Eur J Immunol 1997; 27: 1671, Lipford GB, Sparwasser T, Bauer M, et al., Eur J Immunol 1997; 27: 3420, Chace JH, Hooker NA, Mildenstein KL, Krieg AM, Cowdery JS., Clin Immunol Immunopathol 1997; 84: 185, Roman M, Martin-Orozco E, Goodman J, et al., Nat Med 1997; 3: 849, Sato Y, Roman M, Tighe H, et al., Science 1996; 273: 352, Halpern MD, Kurlander RJ, Pisetsky DS., Cell Immunol 1996; 167: 72). Thus, by inhibiting activation of antigen presenting cells, proinflammatory responses would be blocked.

Cell surface receptors are apparently responsible for DNA uptake into the cell (Yakubov LA, Deeva EA, Zarytova VF, et al., Proc Natl Acad Sci U S A 1989; 86: 6454). Several macrophage cell surface PRR, including scavenger receptor and integins, have been linked to DNA binding and uptake. Kimura et al. reported that the ODN engagement of the scavenger receptor was required for augmented NK cell activity and induction of IFN, although this has been questioned elsewhere (Kimura Y, Sonehara K, Kuramoto E, et al., J Biochem (Tokyo) 1994; 116: 991, Benimetskaya L, Loike JD, Khaled Z, et al., Nat Med 1997; 3: 414). Stein and co-workers determined that Mac-1 (CD11b/CD18) bound ODN and in TNF treated human polymorphonuclear leukocytes (PMNs) induced reactive oxygen species (ROS) generation (Benimetskaya L, Loike JD, Khaled Z, et al., Nat Med 1997; 3:

414). These interactions imply signaling from the cell surface, however, they do not appear to be DNA sequence discriminating.

In a further embodiment the present invention relates to the use of the composition, and/or the oligonucleotide of the present invention, and/or the oligonucleotide to be employed in accordance with the composition of the present invention to inhibit the uptake of DNA by a cell.

In accordance with the present invention it could suprisingly be demonstrated that labeled DNA uptake by macrophages can be blocked at the cell surface with competing G-motif ODN. In non-CpG competitive blocking assays CpG induced signal and cytokine release were blocked, correlating with the need for cellular uptake. In support of this idea Krieg et al. claimed that the CpG receptor site is not on the cell surface because immobilized CpG ODN does not stimulate B cell proliferation (Krieg AM, Yi AK, Matson S, et al., Nature 1995; 374: 546). MacFarlane et al. have claimed that endosomal acidification is a requirement for CpG ODN signaling because inhibitors such as chloroquine blocked CpG ODN rescue of B cells from apoptosis (Macfarlane DE, Manzel L, Krieg AM., Immunology 1997; 91: 586). In agreement with this observation we have shown that these substances without interfering with ODN uptake block signaling and cytokine release in APC. Thus blockade of CpG DNA uptake would block activation of antigen presenting cell proinflammatory responses.

In yet another embodiment the present invention relates to the use of the composition, and/or the oligonucleotide of the present invention, and/or the oligonucleotide to be employed in accordance with the composition of the present invention to co-stimulate cytotoxic T-lymphocytes or natural killer cells.

Cytotoxic cells, e.g. cytolytic T cells or NK cells are responsible for tumor cell and pathogen infected cell removal. The stimulation of either of these immune cells enhances tumor clearance and pathogen clearance.

In a still further embodiment the present invention relates to the use of the composition, and/or the oligonucleotide of the present invention, and/or the

oligonucleotide to be employed in accordance with the composition of the present invention to stimulate natural killer cells.

The present invention also relates to the use of the composition, and/or the oligonucleotide of the present invention, and/or the oligonucleotide to be employed in accordance with the composition of the present invention to enhance the production of antibodies directed against an antigen.

NK cells monitor for the reduction of antigen presenting MHC molecules on the surface of a cell. Often tumors or virally infected cells have reduced MHC molecules on their surface. Thus, NK cells are capable of sensing and killing tumor cells and some virally infected cells. The stimulation of NK cells enhances their surveillance and killing potential and therefore their ability to detect and eliminate tumor cells or virally infected cells.

Furthermore, the present invention relates to the use of the composition, and/or the oligonucleotide of the present invention, and/or the oligonucleotide to be employed in accordance with the composition of the present invention to enhance the uptake of an agent by a cell.

In accordance with the present invention it has surprisingly been found that the oligonucleotides of the present invention may be readily internalized by cells. Thus, it is envisaged that the oligonucleotide of the present invention or the oligonucleotide to be employed in accordance with the composition of the present invention may be utilized as carriers which make possible or facilitate the internalization of an agent by a cell. Said agent may be, e.g., another nucleic acid molecule, a protein, a peptide, a drug, a hormone, a toxin, etc. Furthermore, said agent may be covalently linked to the oligonucleotide of the present invention. Alternatively, after introduction of appropriate interaction domains and/or motifs, the carrier and the agent may also be linked, e.g., via a protein-protein or protein-nucleic acid interaction. If the agent is another nucleic acid molecule, of course, the oligonucleotide of the present invention may also be introduced into said nucleic acid molecule by well known methods. Such methods as well as procedures which may be used to produce, e.g., nucleic acid molecules and/or proteins capable of interacting with each other are described, e.g.,

in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), and Ausubel et al., Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, New York, N.Y. (1989).

In a preferred embodiment of the use of the present invention said agent is a nucleic acid or a (poly)peptide.

In a more preferred embodiment of the use of the present invention said nucleic acid is a gene therapy vector.
Gene therapy, which is based on introducing therapeutic genes into cells by ex-vivo or in-vivo techniques is one of the most important applications of gene transfer. Suitable vectors and methods for in-vitro or in-vivo gene therapy are described in the literature and are known to the person skilled in the art; see, e.g., Giordano, Nature Medicine 2 (1996), 534-539; Schaper, Circ. Res. 79 (1996), 911-919; Anderson, Science 256 (1992), 808-813; Isner, Lancet 348 (1996), 370-374; Muhlhauser, Circ. Res. 77 (1995), 1077-1086; Wang, Nature Medicine 2 (1996), 714-716; WO94/29469; WO 97/00957 or Schaper, Current Opinion in Biotechnology 7 (1996), 635-640, and references cited therein.

In another embodiment the present invention relates to a method for inducing proliferation of bone marrow cells in vitro or in vivo comprising culturing bone marrow cells in the presence of the composition, and/or the oligonucleotide of the present invention, and/or the oligonucleotide to be employed in accordance with the composition of the present invention.

Furthermore, the present invention relates to the use of the composition, and/or the oligonucleotide of the present invention, and/or the oligonucleotide to be employed in accordance with the composition of the present invention to induce proliferation of bone marrow cells in vitro or in vivo.

In a preferred embodiment of the method or the use of the present invention said bone marrow cells are macrophage precursor cells.

In the bone marrow, progenitors for all lineages of the cells of the immune system are generated. Descending from pluripotent stem cells progenitors proliferate and differentiate under the influence of hematopoietic growth factors and cytokines. Agents that interfere with the cell cycle or change levels of hematopoietic cytokines have therefore profound effects on the homeostasis of cells generated in the bone marrow. For example, treatment of malignant neoplasias with cytostatic drugs is often limited by severe leukopenia. On the other hand, administration of colony stimulating factors like G-CSF leads to enhanced proliferation of granulocyte precursors and their mobilization into peripheral blood. Situations where cytokines are produced systemically also influence the production of blood cells in the bone marrow and the relative lineage distribution.

Given the importance of the bone marrow for the generation of blood and immune cells, it was surprisingly found in accordance with the present invention that the oligonucleotide of the present invention, and/or the oligonucleotide to be employed in accordance with the composition of the present invention can be advantageously used to induce proliferation of bone marrow cells. In this regard it has to be noted that up to the making of the present invention it was not possible to cultivate macrophage precursor cells in vitro. This is mainly due to the fact that all agents that were available so far and suitable for the propagation of macrophage precursor cells in vitro either themselves also induce differentiation of said precursor cells or induce the production of differentiation stimulating cytokines, thereby obviating further proliferation of said precursor cells.

In contrast, it was found in accordance with the present invention that the oligonucleotides of the present invention induce neither differentiation of said precursor cells nor the synthesis of the above mentioned cytokines. Thus, the present invention enables for the first time the person skilled in the art to propagate macrophage precursor cells in vitro.

It is also envisaged in accordance with the present invention to take advantage of the oligonucleotides of the present invention to stimulate and/or enhance the growth of bone marrow cells in vivo. Advantageously, the oligonucleotides of the present

invention do not display the above mentioned and other adverse side effects of cytokines used up to now in such methods and, thus, lead to a substantial and unexpected improvement of the same.
In addition, it is envisaged in accordance with the invention to use the oligonucleotides of the invention in combination with cytokines and/or growth factors.

In a still further embodiment the present invention relates to the use of the composition, and/or the oligonucleotide of the present invention, and/or the oligonucleotide to be employed in accordance with the composition of the present invention to induce apoptosis in tumor cells.

The references cited in this application are incorporated herewith by reference.

The figures show:

Figure 1:
The oligonucleotides of the present invention fail to induce TNF and inhibit TNF production induced by immunostimulatory DNA.

Figure 2:
The oligonucleotides of the present invention inhibit TNF secretion induced by immunostimulatory DNA.

Figure 3:
The oligonucleotides of the present invention block entry of bacterial DNA into macrophages and thus block CpG induced signaling.

Figure 4:
Inhibition of macrophage signal transduction by blockade of CpG ODN.

Figure 5:
The oligonucleotides of the present invention prevent lethal shock induced by immunostimulatory DNA. Sequence specificity and dose dependency.

Figure 6:

The oligonucleotides of the present invention do not interfere with Superantigen- or LPS-induced lethal cytokine syndrome.

Figure 7:
The oligonucleotides of the present invention act as adjuvants for generation of antigen-specific cytotoxic T cells in vivo.

Figure 8:
The oligonucleotides of the present invention induce NK activity in vivo.

Figure 9:
Sequence dependency of T cell costimulation induced by the oligonucleotides of the present invention.

Figure 10:
Single stranded oligonucleotides but not double-stranded or complementary oligonucleotides costimulated T cells.

Figure 11:
Proliferation of mouse splenocytes and primary BMC in response to an oligonucleotide of the present invention.

Figure 12:
Restimulation of BMC cultured for six days with an oligonucleotide of the present invention.

Figure 13:
BMC stimulated with an oligonucleotide of the present invention are responsive to M-CSF and GM-CSF, but not to G-CSF.

Figure 14:
Sequence specificity of the G-motif binding protein.

Figure 15:

UV-crosslink analysis of binding of single-stranded oligonucleotides of the present invention to protein extracts prepared from J774 cells.

The examples illustrate the invention.

The term "G-motif ODN" as used throughout the examples denotes the oligonucleotides of the present invention and/or the oligonucleotides to be employed in accordance with the composition of the present invention.

Example 1: G-motif ODN fail to induce TNF and inhibit TNF production induced by immunostimulatory DNA.

The macrophage cell line J774 was seeded at 10^5/culture and stimulated with the reagents indicated in Figure 1A. After 12 h the cell culture supernatant was removed and tested for TNF content by ELISA. The results in Figure 1A demonstrate that LPS and the immunostimulatory CpG-motif ODN (1668) induced TNF secretion. G-motif ODN PZ1, PZ2 and PZ3 failed to induced TNF. The control ODN PZ4, PZ5 or poly-G were also ineffective.

The macrophage cell line J774 was seeded at 10^5/culture and stimulated with the reagents indicated in Figure 1B. To cultures that were stimulated with ODN 1668 at 1µM, different amounts of PZ-ODN were added. After 12 h the cell culture supernatant was removed and tested for TNF content by ELISA. The results shown in Figure 1B demonstrate that the ODN PZ1, PZ2 and PZ3 inhibited the TNF inducing capacity of ODN 1668 even at very low concentrations. ODN PZ4 and PZ5 and poly-G were only effective at high concentrations.

Example 2: G-motif ODN inhibit TNF secretion induced by immunostimulatory DNA.

The macrophage cell line J774 was seeded at 10^5/culture and stimulated with the

reagents indicated in Figure 2. After 6 h the cell culture supernatant was removed and tested for TNF content by ELISA.

The dose response experiment shown in Figure 2A demonstrates that ODN PZ1, PZ2 and PZ3 inhibit TNF secretion induced by the immunostimulatory ODN 1668 even at very low concentrations.

As shown in Figure 2B, the ODN PZ1 and PZ2 were added to cultures stimulated with ODN 1668 at the time points and concentrations indicated. The results reveal that addition of G-motif ODN even after 120 min reduced TNF secretion, i.e. inhibition with PZ ODN can occur post CpG-motif stimulation

Figure 2C shows the results of an experiment performed to investigate the time kinetics of G-motif ODN induced inhibition of TNF secretion. The macrophage cell line J774 was seeded at 10^5/culture and stimulated with the reagents indicated. The G-motif ODN PZ1, PZ2 and PZ3 were added as inhibitors at the indicated time. After 6 h the cell culture supernatant was removed and tested for TNF content by ELISA. As a control the content of cultures stimulated with 1668 only was removed after the incubation periods indicated (0.5 to 6 h). The results indicate, that PZ ODN suppress TNF production up to 4 hours after initiation of the cultures by acutely stopping TNF secretion. One should compare the TNF amount detected in the 1668 plus PZ ODN conditions versus the 6h time point for the 1668 only condition.

Example 3: G-motif ODN block entry of bacterial DNA into macrophages and thus block CpG induced signaling.

Macrophages uptake labeled CpG-motif ODN and this is postulated to be needed for efficient cellular activation. Macrophages were incubated with 1µM FITC-CpG ODN, plus or minus a G-motif ODN, at 37^0C for 2h and visualized by fluorescence microscopy. Panel A of Figure 3 depicts FITC-CpG ODN localization. Panel B depicts FITC-CpG ODN following a preincubation of the cells with 3 µM G-motif ODN. Panel C represent the kinetics of biotinylated CpG-motif ODN uptake into macrophages, plus (squares) or minus (circles) G-motif ODN as inhibitor. It can thus be shown that G-motif ODN effectively inhibits uptake of DNA into cells.

Example 4: Inhibition of macrophage signal transduction by blockade of CpG ODN.

The macrophage cell line, RAW-264 was stably transfected with vectors which utilized a NFkB response element (panel A), the TNF-a promoter (panel B) or the IL-12 p40 promoter (see panel C of Figure 4) driving the expression of a luciferase gene. The cells were stimulated for 3 h with CpG ODN (closed bars) or LPS (hatched bars) to induce luciferase activity measured as arbitrary light units in the presence or absence of a G-motif ODN. In panel C of Figure 4, N.T. represents „not tested" because RAW 264 cells do not induce IL-12 p40 promoter activity in response to LPS. The results are given as percent control which represents the mean arbitrary light units in the presence of inhibitor divided by the uninhibited reading. The values represent mean and standard deviations of 3 determinations.

These results demonstrate that blockade with G-motif ODN inhibits macrophage responses to foreign DNA at the promoter level. This includes reduction of NFkB activity, a critical transcription factor which promotes the induction of many inflammatory responses. TNF promoter activity is also reduced, in compliance with the cytokine release data (Figure 1 and 2). In addition the promoter activity for IL-12 is reduced, a critical cytokine needed for Th1 induction.

Example 5: G-motif ODN prevent lethal shock induced by immunostimulatory DNA. Sequence specificity and dose dependency.

To induce lethal shock by immunostimulatory DNA, BALB/c mice were injected with 5 nmol of ODN 1668 plus D-galactosamine (D-GalN) i.p. (closed circles in Figure 5A). The number of surviving mice is shown. One group of mice received in addition the G-motif ODN PZ2 (closed diamonds in Figure 5A) or a non G-motif ODN control (PZ5) (closed squares in Figure 5A) at 5 nmol.

In another experiment lethal shock was induced by immunostimulatory ODN 1668 at 5 nmol and D-GalN (closed circles in Figure 5B). Titrated amounts of G-motif ODN PZ2 were injected simultaneously (0.2 nmol, open squares; 1 nmol closed squares; 5 nmol, closed diamonds in Figure 5B) and the number of surviving mice were recorded.

As shown in Figure 5C G-motif ODN prevent lethal shock induced by bacterial DNA. C3H-HeJ mice were injected i.p. with 300 µg bacterial DNA (E. coli) plus D-galactosamine (D-GalN) (closed circles) to induce lethal shock. As control DNA derived from calf thymus was used (open circles). The G-motif ODN PZ31 (defined in Table 2) by itself did not induce lethal shock (open triangle), yet ODN 31 completely prevented lethal shock induced by bacterial DNA when injected simultaneously (closed triangles down). A second G-motif ODN, ODN 35, (closed triangles up) also improved survival, however a control non-G-motif ODN, PZ332 (closed squares) failed to prevent lethal shock.

These experiments demonstrate that G-motif ODN prevent lethal shock induced by foreign DNA in a sequence dependent and dose-dependent fashion.

Example 6: G-motif ODN do not interfere with Superantigen- or LPS-induced lethal cytokine syndrome.

To provoke a lethal cytokine syndrome (lethal shock) by the superantigen staphylococcal enterotoxin B (SEB), mice were injected i.p. with 10 µg SEB and 20 mg D-galactosamine (D-GalN) into BALB/c mice. The number of surviving mice is shown in Figure 6A (open circles). It was then tested whether the G-motif ODN PZ2 would be able to interfere with the superantigen-induced lethal shock. To this PZ2 was injected with SEB at 10 nmol / mouse i.p.(closed circles in Figure 6A).

LPS (Endotoxin)-mediated shock was induced by injecting 10 µg LPS and 20 mg D-GalN into BALB/c mice (closed circles in Figure 6B). To test whether G-motif ODN would interfere with LPS-induced lethal cytokine syndrome, mice received in addition 10 nmol of ODN PZ2 (open squares in Figure 6B). The number of surviving mice was recorded.

The experiments show, that G-motif ODN fail to interfere with lethal shock syndrome induced by either Superantigens (A) or Endotoxin (B).

Example 7: G-motif ODN act as adjuvants for generation of antigen-specific cytotoxic T cells in vivo.

C57Bl/6 mice were injected with liposome entrapped Ovalbumin (Ova) into the footpad. One group of mice received 10 nmol of ODN PZ2 in addition (see Figure 7B). After four days the draining lymph nodes were removed and the cells prepared. They were then cultured at 3 x 10^6 in 2 ml tissue culture plates with 10U/ml recombinant IL-2. After four days the cells were harvested and tested in a ^{51}Cr-release assay for cytolytic activity against syngeneic target cells pulsed with either the immunodominant Ova-peptide or a third party peptide (VSV).
The results shown in Figure 7 demonstrate that the ODN PZ2 acts as an adjuvants during the generation of specific cytolytic T cells.

Example 8: G-motif ODN induce NK activity in vivo.

C57Bl/6 mice were injected with 10 nmol of ODN PZ2 into the footpad. After four days the draining lymph nodes were removed and the cells prepared. They were then cultured at 3 x 10^6 in 2 ml tissue culture plates with 10U/ml recombinant IL-2. After four days the cells were harvested and tested in a ^{51}Cr-release assay for cytolytic activity against NK-sensitive YAC-1 target cells. Prior to the cytotoxicity assay from the effector population $CD4^+$ cells (closed triangle up in Figure 8), $CD8^-$ cells (closed triangle down in Figure 8) or both T cell populations (closed diamond in Figure 8) were removed by magnetic cell sorting.
The results demonstrate that PZ2 induces NK-activity in vivo. The NK effector cell is CD4 and CD8 negative and resembles a classical NK cell population.

Example 9: Sequence dependency of T cell costimulation induced by G-motif ODN.

T lymphocytes from lymph node cells from C57 Bl/6 mice were negatively selected. To accomplish this cells were first passed over a Sephadex G10 column to remove macrophages and activated lymphocytes. Then the cells were incubated with magnetic beads coupled with anti-mouse IgG and the IgG^+ B cell population was

removed by magnetic cell sorting. The purity of the T cell population exceeded > 97% as determined by cytometrical analyses.

Purified T cells were stimulated in cell culture medium containing 10% FCS, antibiotics and indomethacin. Cultures were set up in u-shaped microtiter plates that had been precoated with anti-CD3 monoclonal hamster antibodies. To this plates were incubated with 10 µg rabbit anti hamster polyclonal antibodies in PBS for 16 h. Thereafter the plates were washed with PBS and the plastic surface was blocked by addition of 2% BSA for 4 h. After additional washing 1µg/ml anti-CD3 monoclonal antibody in PBS was added. After incubation for 16h plates were again washed and then used for the cultures. 15,000 selected T cells were added to each culture. Controls included supplementation with IL-2 (10U/ml), ConA (1µg/ml) or LPS (10µg/ml). Oligonucleotides were added at 5 µM, 1 µM or 0.2µM. Four replicate cultures each were set up. After 4 days 100 µl of the culture supernatant was collected and tested for IFN-gamma content by ELISA (see Figure 9B). The cultures were then pulsed with ^{3}H-thymidine and the proliferative response was recorded (Figure 9A).

To analyze the effects of ODN on T cells a costimulation assay was used. In this assay purified T cells are stimulated via their TCR (signal 1). This signal is not sufficient to induce cytokine secretion and subsequent T cell growth (see Figure 9A). Addition of exogenous IL-2 demonstrate that signal 1 is operative. ODN by itself have no stimulatory activity on T cells alone. If however T cells receive a signal via their TCR they become sensitive to ODN. ODN provide to these T cells a potent second signal that induces cytokine secretion (Figure 9B) and T cell growth (Figure 9A). The results demonstrate that G-motif ODN-costimulated anti-CD3 triggered T cells in a sequence and concentration dependent fashion. The analyses allowed the definition of the minimal ODN motif effective for T cell costimulation.

Example 10: Single stranded G-motif ODN but not double-stranded or complementary ODN costimulated T cells.

T lymphocytes from lymph node cells from C57 Bl/6 mice were negatively selected. To this cells were first passed over a Sephadex G10 column to remove

macrophages and activated lymphocytes. Then the cells were incubated with magnetic beads coupled with anti-mouse IgG and the IgG^+ B cell population was removed by magnetic cell sorting. The purity of the T cell population exceeded > 97% as determined by cytometrical analyses.

Purified T cells were stimulated in cell culture medium containing 10% FCS, antibiotics and indomethacin. Cultures were set up in u-shaped microtiter plates that had been precoated with anti-CD3 monoclonal hamster antibodies. To this plates were incubated with 10 µg rabbit anti hamster polyclonal antibodies in PBS for 16 h. Thereafter the plates were washed with PBS and the plastic surface was blocked by addition of 2% BSA for 4 h. After additional washing 1µg/ml anti-CD3 monoclonal antibody in PBS was added. After incubation for 16h plates were again washed and then used for the cultures. 15,000 selected T cells were added to each culture. Controls included supplementation with IL-2 (10U/ml), or LPS (10µg/ml). Oligonucleotides were added at 5 µM, 1 µM or 0.2µM. 4 replicate cultures each were set up. After 4 days the cultures were pulsed with ^{3}H-thymidine and the proliferative response was recorded.

The experiment shown in Figure 10A demonstrates that the G-motif ODN PZ1, PZ2 and PZ3 but not the controls PZ4 or PZ5 costimulate T cells. The DNA-complementary derivatives of PZ1K, PZ2K and PZ3K are negative. ODN 1628 (a CpG-ODN) was used as a positive control. In addition, poly G or poly A ODN do not stimulate T cells.

The experiment shown in Figure 10B details that the G-motif ODN are only active in single stranded form (PZ1, PZ2 and PZ3). The double-stranded form (PZ1, PZ2 and PZ3 plus PZ1K, PZ2K and PZ3K, respectively) fail to costimulated T cells. In addition poly G does not stimulate T cells.

Example 11: Proliferation of mouse splenocytes and primary BMC in response to a G-motif ODN.

4×10^4 cells were cultured in Click's RPMI containing 5 % FCS in the presence of the indicated concentrations of G-motif ODN GR1 (TTGGAGGGGGTGGTGGGG) or

CpG-motif 1668. ^{3}H-thymidine incorporation was determined by a 6-hour pulse at days 2, 4 and 6.

These experiments, which are shown in Figure 11, indicate that a G-motif ODN induces a proliferative response in the bone marrow and that this response is qualitatively different from that observed with a CpG-motif ODN.

Example 12: Restimulation of BMC cultured for six days with a G-motif ODN.

Primary mouse BMC were cultured in Click's RPMI containing 5 % FCS for six days in the presence of ODN GR1 or 1668. After harvesting and washing of the cells, 2 x 10^4 were reseeded in 96-well flat-bottomed plates and cultured additional five days with medium alone or in the presence of the indicated stimuli, followed by a ^{3}H-thymidine pulse of 6h.

These experiments, which are shown in Figure 12, indicate that a G-motif ODN induces a renewable proliferative response in the bone marrow and that this response is qualitatively different from that observed with a CpG-motif ODN. This information implies that a renewable precursor population was expanding when stimulated with G-motif ODN. However this implies that differentiation does not occur as may be the case with CpG ODN stimulation

Example 13: G-motif stimulated BMC are responsive to M-CSF and GM-CSF, but not to G-CSF.

After seven days of culture in the presence of G-motif ODN, cells were harvested, washed and reseeded at a density of 2 x 10^4 cells per well in a 96-well plate. M-CSF, GM-CSF, G-CSF and ODN GR1 were added at different concentrations. After 60 h, ^{3}H-thymidine was added to the plates for 6h.

These experiments, which are shown in Figure 13, indicate that G-motif ODN expand a myeloid precursor from bone marrow. The cytokine addition serves as a growth stimulus and induces differentiation. Because of the susceptibility of the precursor population to M-CSF and not G-CSF it can be implied that G-motif expanded precursors are myeloid in nature. Myeloid precursor expansion and differentiation

would lead to enhanced antigen presentation capacity and thus ability to defend against pathogen infection.

Example 14: Sequence specificity of the G-motif binding protein.

Extracts of J774 cells were incubated with single-stranded, radiolabeled GR1 for 30 min at room temperature. Different G-motif and non-G-motif containing competitors were added in a 150-fold molar excess. Treatment with Proteinase K or RNAseA was performed before the binding reaction was initiated. Free probe was separated from complexes formed by running a 5 % non-denaturing PAGE.

Sequences of competitors:

G-motif containing ODN

GR1	TTGGA**GGGGG**TG**GTGGG**G
EGR1	AGC**GGGGG**CGAGC**GGGGG**CG
SP1	TCGAT**CGGGG**CG**GGGCG**AGC

Non-G-motif containing ODN

Purα	AAAAGGGAAGGGATGGCT
PurαOri	GGAGGCGGAGGCGGAGGCGGAGGC
1668	TCCATGACGTTCCTGATGCT
NFkB	ATATAAGGGAAATTTCCAGC
GR1comp	CCCCACCACCCCCTCCAA

As can be seen in Figure 14, a characteristic double banding pattern can be detected which is specific for G-motif ODN. Although Purα, PurαOri and NFkB ODN have very close approximations to the G-motif neither are capable of blocking the labeled G-motif ODN from binding its target. The target is a protein or protein as demonstrated by its loss upon proteinase K treatment. However RNAse A failed to destroy the target, thus the target is not RNA as would be the case for antisense ODN technologies.

Example 15: UV-crosslink analysis of binding of single-stranded G-motif ODN to protein extracts prepared from J774 cells.

After incubation in hypotonic buffer, J774 cells were lysed by douncing, followed by pelleting of the nuclei. The supernatant was transferred to a new tube and cleared of insoluble material by a 30 min centrifugation at 35,000 x g. 5 µg of the extract was incubated for at room temperature for 30 min with 3 ng of ^{33}P-labeled G-motif ODN. To reduce non-specific binding 1 µg of polydIdC was added to the binding reaction. Specific competitors; G-motif, GR1 and CpG-motif, 1668, were included at a 100-fold molar excess. Subsequently, samples were exposed on ice to UV-light, followed by electrophoresis on a 12.5 % SDS-PAGE. The dried gel was exposed overnight on a phosphoimager. The size of shifted bands was calculated from the positions of the rainbow molecular weight markers.

Figure 15 shows that two protein bands become detectable by this method, at approximately 58kD and 25kD. These binding protein are apparently non-nuclear in origin and are specific for the G-motif similar to the protein target detected and discussed in Example 14, supra.

Table 1: Prototypic blocking oligonucleotides

PZ1	5'	CTCCTAGCGGGGGCGTCCTAT	3'
PZ2	5'	CTCCTAG**T**GGGGG**T**GTCCTAT	3'
PZ3	5'	CTCCTA**TT**GGGGG**TT**TCCTAT	3'
PZ4	5'	CTCCTA**GT**GG**TT**G**TG**TCCTAT	3'
PZ5	5'	CTCCTA**GTTGTTTTG**TCCTAT	3'
Poly-G	5'	**GGGGGGGG**GGGGG**GGGGGGGG**	3'

Bold lettering represents changed nucleotides from the initial PZ1 ODN.

Table 2: Sequences for definition of highest affinity antagonist of the surface receptor

To define the high affinity blocking motif, the sequence PZ3 was iteratively modified in the core G-motif. These sequences were tested through a blocking titration range

similar to fig. 1B and fig.2A. A determination of the 50% inhibitory concentration (IC50) is given for each ODN.

Table 2A: Data arrange to easily show the iterative process. Last column of values from preliminary experiment

				IC_{50} (nM)	IC_{50} (nM)
PZ31 (PZ3)	5'	CTCCTATTGGGGGTTTCCTAT	3'	80.3	58.7
PZ32	5'	CTCCTATTGGGG**T**TTTCCTAT	3'	187.2	182.0
PZ33	5'	CTCCTATTGGG**T**GTTTCCTAT	3'	516.0	138.5
PZ34	5'	CTCCTATTGG**T**GGTTTCCTAT	3'	1382.1	495.4
PZ35	5'	CTCCTATTG**T**GGGTTTCCTAT	3'	97.3	67.6
PZ36	5'	CTCCTATT**T**GGGGTTTCCTAT	3'	116.8	32.2
PZ37	5'	CTCCTATTGGG**TT**TTTCCTAT	3'	647.0	
PZ38	5'	CTCCTATTGG**T**G**T**TTTCCTAT	3'	1003.0	
PZ39	5'	CTCCTATTG**T**GG**T**TTTCCTAT	3'	916.0	
PZ310	5'	CTCCTATT**T**GGG**T**TTTCCTAT	3'	344.6	
PZ311	5'	CTCCTATTGG**TT**GTTTCCTAT	3'	1092.9	
PZ312	5'	CTCCTATTG**T**G**T**GTTTCCTAT	3'	1392.1	
PZ313	5'	CTCCTATT**T**GG**T**GTTTCCTAT	3'	985.4	
PZ314	5'	CTCCTATTG**TT**GGTTTCCTAT	3'	2075.6	
PZ315	5'	CTCCTATT**T**G**T**GGTTTCCTAT	3'	2230.3	
PZ316	5'	CTCCTATT**TT**GGGTTTCCTAT	3'	684.0	
PZ332	5'	CTCCTATT**TTTTT**TTTCCTAT	3'	>5000.0	

Table 2B: Data arrange to easily show the affinity rank order of the oligonucleotides of the present invention. Range of affinity differences from highest too lowest greater than 62 fold. These data combined with the uptake data (Fig. 3) demonstrate that a sequence selective receptor on the surface of cells is responsible for DNA uptake. Because the receptor is sequence selective high affinity interacting oligonucleotides can be designed which interfere with the potential inflammatory effects of CpG-motif containing DNA.

				IC_{50} (nM)
PZ31 (PZ3)	5'	CTCCTATTGGGGGTTTCCTAT	3'	80.3
PZ35	5'	CTCCTATTG**T**GGGTTTCCTAT	3'	97.3
PZ36	5'	CTCCTATT**T**GGGGTTTCCTAT	3'	116.8
PZ32	5'	CTCCTATTGGGG**T**TTTCCTAT	3'	187.2
PZ310	5'	CTCCTATT**T**GGG**T**TTTCCTAT	3'	344.6
PZ33	5'	CTCCTATTGGG**T**GTTTCCTAT	3'	516.0
PZ37	5'	CTCCTATTGGG**TT**TTTCCTAT	3'	647.0
PZ316	5'	CTCCTATT**TT**GGGTTTCCTAT	3'	684.0
PZ39	5'	CTCCTATTG**T**GG**T**TTTCCTAT	3'	916.0
PZ313	5'	CTCCTATT**T**GG**T**GTTTCCTAT	3'	985.4
PZ38	5'	CTCCTATTGG**T**G**T**TTTCCTAT	3'	1003.0
PZ311	5'	CTCCTATTGG**TT**GTTTCCTAT	3'	1092.9
PZ34	5'	CTCCTATTGG**T**GGTTTCCTAT	3'	1382.1
PZ312	5'	CTCCTATTG**T**G**T**GTTTCCTAT	3'	1392.1
PZ314	5'	CTCCTATTG**TT**GGTTTCCTAT	3'	2075.6
PZ315	5'	CTCCTATT**T**G**T**GGTTTCCTAT	3'	2230.3
PZ332	5'	CTCCTATT**TTTTT**TTTCCTAT	3'	>5000.0 [a]

a. greater than calculable range for assay

Table 3: Utilizing the motif GNGGG or GGGNG a determination of rank order of replacement nucleotides for N

				IC_{50} (nM)
PZ31 (PZ3)	5'	CTCCTATTGGGGGTTCCCTAT	3'	340.2
PZ35	5'	CTCCTATTG**T**GGGTTCCCTAT	3'	289.8
PZ35A	5'	CTCCTATTG**A**GGGTTCCCTAT	3'	247.0
PZ35C	5'	CTCCTATTG**C**GGGTTCCCTAT	3'	994.3
PZ33	5'	CTCCTATTGGG**T**GTTCCCTAT	3'	488.2
PZ33A	5'	CTCCTATTGGG**A**GTTCCCTAT	3'	649.0
PZ33C	5'	CTCCTATTGGG**C**GTTCCCTAT	3'	1122.5

Table 4: The effect of addition G flanking the motif lowers blocking affinity

				IC_{50} (nM)
PZ31 (PZ3)	5'	CTCCTATTGGGGGTTCCCTAT	3'	76.1
PZ31-G9	5'	CTCCTA**GG**GGGGG**GG**CCCTAT	3'	377.6
PZ31-G13	5'	CTCC**GGGG**GGGGG**GGGG**CTAT	3'	2050.1
PZ31-G17	5'	CT**GGGGGG**GGGGG**GGGGGG**AT	3'	3178.3
Poly G	5'	**GGGGGGGG**GGGGG**GGGGGGGG**	3'	1568.2

Table 5: The effect of random nucleotides flanking the motif increases blocking affinity. Blocking affinity was minimally effected by position of the motif, however the G-motif at the 3'end had the greatest affinity

				IC_{50} (nM)
PZ31 (PZ3)	5'	CTCCTATT**GGGGG**TTTCCTAT	3'	76.1
PZ31-Random	5'	HHHHHHHW**GGGGG**HHHHHHHH	3'	11.6
PZ31-Random-5'	5'	**GGGGG**HHHHHHHHHHHHHHHH	3'	<10.0
PZ31-Random-3'	5'	HHHHHHHHHHHHHHHW**GGGGG**	3'	<3.0

H= A,T or C

W= A or T (W was used if preceding a G to avoid the CpG motif)

Table 6: Minimal length needed for high affinity block with G-motif flanked by random nucleotides

				IC_{50} (nM)
PZ31 (PZ3)	5'	HHHHHHHW**GGGGG**HHHHHHHH	3'	76.1
PZ31-17	5'	HHHHW**GGGGG**HHHHH	3'	67:8
PZ31-13	5'	HHHW**GGGGG**HHH	3'	570:0
PZ31-9	5'	HW**GGGGG**HH	3'	>3000.0
PZ31-5	5'	**GGGGG**	3'	>3000.0

H= A,T or C

W= A or T (W was used if preceding a G to avoid the CpG motif)